Attorney's Docket No.: US 1292/01(VA)

GLUCOSE SENSITIVE REGULATOR OF INSULIN TRASNCRIPTION

PETER M. THULÉ, M.D.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority on prior U.S. Provisional Application S.N. 60/239,113, filed October 11, 2000, and which is incorporated herein in its entirety by reference.

REFERENCE TO SEQUENCE LISTING

Sub A1

~~**[0002]** The present application incorporates by reference SEQ ID NO.: 1, SEQ ID NO.: 2, SEQ ID NO. 3, SEQ ID NO.: 4, SEQ ID NO.: 5 and SEQ ID NO.: 6 provided herewith on a diskette, created on October 5, 2001 and containing 3,728 bytes. The information recorded on the diskette is identical to the written sequence listing provided herein.~~

BACKGROUND OF THE INVENTION

[0003] Type 1 diabetes mellitus (DM) is usually precipitated by autoimmune destruction of pancreatic β-cells, leading to insufficient insulin production (Reference 1). Since clinical symptoms are caused by diminished production of a single protein, diabetes is a natural candidate for treatment by gene therapy. The basic components of insulin gene therapy are widely available. Functional insulin genes can be transferred to multiple tissues (References 2-4), and the capacity of non-β-cells to secrete biologically active transgenic insulin in sufficient quantities to affect metabolism is well established (References 2-6). Multiple investigators have demonstrated functional insulin gene transfer both *in vitro*, and *in vivo* (References 2 and 7-8). However, attempts to regulate transgenic insulin production have proven inadequate (References 9-10). Consequently, in a variety of insulin gene transfer protocols secretion of transgenic insulin has been either insufficient to normalize blood glucose (References 2-5 and 10), has affected glycemia only moderately, or for short periods of time (References 4, 5 and 10-14), or has produced lethal hypoglycemia (References 2-3 and 8). Thus, for insulin gene therapy to be effective, it is widely accepted that insulin production must be regulated.

[0004] The critical importance of regulated transgenic insulin production was underscored by the work of Muzzin, et al. They successfully

induced insulin production from the livers of STZ-treated rats by administering a retrovirus containing an insulin transgene (Reference 3). Transgenic insulin secretion was sufficient to prevent diabetic ketoacidosis, while permitting the animals to gain weight. Moreover, they avoided lethal hypoglycemia in a subset of animals by limiting vector dosage. However, reductions in vector sufficient to enable survival with prolonged fasting produced hyperglycemia when the animals were fed, presumably because transgenic insulin production could not increase to meet expanded demand (Reference 3). Others have demonstrated transgenic insulin secretion that is regulated by cAMP, glucocorticoids, insulin, or glucose by utilizing metabolically sensitive promoters in hepatocytes or hepatoma cells, and Simpson et al have demonstrated glucose responsiveness in insulin expressing HepG2 cells (References 15-18). However, transfer of these regulatory mechanisms to *in vivo* models has been difficult (Reference 9). We have overcome these limitations, and have developed a glucose and insulin sensitive promoter capable of appropriately coupling metabolic requirements for insulin, with insulin production from the liver of a diabetic animal.

OBJECTS AND SUMMARY OF THE INVENTION

[0005] An object of the present invention is to provide a glucose sensitive regulator of insulin transcription that regulates the transcription of an insulin expression sequence that has been introduced into liver-parenchymal cells (hepatocytes), or well-differentiated hepatoma cell lines.

[0006] Another object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein transcription of an insulin expression sequence is stimulated by exposure of these hepatocytes, either *in vitro* or *in vivo*, to glucose, but not other carbohydrates, such as lactate.

[0007] Yet another object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein transcription of an insulin expression sequence that is stimulated by glucose is inhibited by exposure of these hepatocytes to glucagon, and possibly other stimulators of cyclic-adenosine mono-phosphate within hepatocytes.

[0008] Still yet another object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein transcription is stimulated by exposure of cells to glucocorticoids.

[0009] An additional object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein the transcription is inhibited, either *in vitro* or *in vivo*, by exposure of hepatocytes to stimulators of

the insulin receptor, including, but not limited to insulin from beef, pigs, humans, and rats, and probably including non-protein insulinomimetics.

[0010] Yet an additional object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein the combination of the aforementioned effects provides sufficient coupling between the actual metabolic requirement for insulin-action in, and the production of insulin-action as to maintain near euglycemia in diabetic animals, whether they are enduring a short (24-hours or less) fast, are subjected to large carbohydrate loads (i.e., a glucose-tolerance test), or are feeding *ad libitum*.

[0011] Still yet an additional object of the present invention is to provide a glucose sensitive regulator of insulin transcription which when coupled to an insulin expression sequence, provides sufficient regulation of insulin production to inhibit pathologic ketogenesis and the development of diabetic ketoacidosis in diabetic animals, and that this capacity is believed to be reproducable in humans.

[0012] Still yet an additional object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein the sum effect of glycemic control in animals, and by inference, people, treated with an insulin gene controlled by the invention is an inhibition of the processes leading to long-term complications in subjects with diabetes mellitus. Such complications include, but are not limited to, microvascular disease,

macrovascular disease, serum lipid abnormalities, neuropathies, myopathies, and coagulopathies.

[0013] Another object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein the transcription of a transgene by the invention is minimal in non-hepatocyte, or non-hepatoma, or poorly-differentiated hepatoma cell lines, with the possible exception of certain kidney or small bowel, or endometrial cells.

[0014] Yet another object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein intracellular production of insulin within hepatocytes, mediated via the invention, may exert effects separate and distinguishable from those produced by secreted insulin. These effects may include the inhibition of cellular protein-degradation machinery, and either inhibitory or stimulatory effects on the actions of intracellular hormone receptors of the glucocorticoid, Vitamin D, thyroid-hormone, estrogen, progesterone, and androgen receptor class.

[0015] Still yet another object of the present invention is to provide a glucose sensitive regulator of insulin transcription wherein hepatic insulin production is believed to be capable of reducing protein catabolism during short term fasting, and may reduce protein catabolism in other catabolic conditions.

[0016] In summary, the present invention provides a hepatocyte specific promoter/regulator/transgene whose transcriptional activity is stimulated

by glucose and inhibited by insulin. Application of this promoter in hepatocytes produces insulin transgene expression that is stimulated by exposure to glucose, and inhibited by exposure of cells to insulin. Glucocorticoids stimulate promoter activity independently of, and synergistically to glucose, while glucagon interferes with glucose stimulation. Glucose- and insulin-sensitive promoters were constructed by inserting glucose-responsive elements (GIRE's) from the rat L-pyruvate kinase (L-PK) gene into the insulin-sensitive, liver specific, rat insulin-like growth factor binding protein-1 (IGFBP-1) promoter. Glucose (5 to 25 mM) stimulated, and insulin (10^{-10} to 10^{-7}M) inhibited, reporter gene expression driven by these promoters in primary cultured rat hepatocytes. The capacity of transfected hepatocytes to secrete mature, biologically active insulin was demonstrated using a human proinsulin cDNA (2xfur), modified to allow protein processing by endogenous endopeptidase activity. Medium conditioned by insulin-producing hepatocytes contained greater than 300μU/ml immunoreactive insulin, while denaturing SDS-PAGE of an anti-insulin immunoprecipitate revealed bands with the mobilities of insulin A, and B-chains. Biological activity of hepatocyte-produced insulin was demonstrated in a transfection assay, in which medium conditioned by insulin-producing hepatocytes exerted an effect similar to 10^{-7}M insulin. The glucose and insulin sensitive promoter was then combined with the modified human pro-insulin cDNA to create a metabolically sensitive insulin transgene [$(GIRE)_3$BP-1 2xfur]. In both H4IIE hepatoma cells stably transfected with this construct, and normal rat hepatocytes, $(GIRE)_3$BP-1

2xfur mediated insulin secretion increased in response to stimulation by glucose. Moreover, a capacity to decrease insulin production in response to diminishing glucose exposure was also demonstrated.

[0017] To demonstrate application of the transgene of the invention, for the treatment of diabetes mellitus *in vivo*, a recombinant adenovirus vector, Ad/(GIRE)$_3$BP-1 2xfur, was administered to rats made diabetic with streptozotocin. The hepatic expression of transgenic insulin was verified by RT-PCR, and confirmed glucose responsive stimulation of transgenic insulin secretion *in vivo* by serum RIA. Following a portal system injection of either Ad/(GIRE)$_3$BP-1 2xfur, or an empty adenoviral vector, animals made diabetic with either low (120mg/kg), or high (290mg/kg) dose streptozotocin (STZ) were monitored for changes in body weight, and blood glucose. Without subcutaneous insulin injections, blood glucose values of sham-treated animals (n=8) remained elevated, and animals failed to gain weight (n=4), or died (n=4). In contrast, body weight of Ad/(GIRE)$_3$BP-1 2xfur-treated animals (n=13) increased, and blood glucose remained at near normal levels from one to twelve weeks. Glucose values <50mg/dl were infrequently observed, and no Ad/(GIRE)$_3$BP-1 2xfur-treated animal succumbed to hypoglycemia. Treatment with the insulin transgene enabled diabetic animals to reduce blood sugars following a glucose load, and to maintain blood sugar levels during a 10-hour fast. Hepatic production of human insulin produced

near normal glycemia, and weight gain, without exogenous insulin, and without lethal hypoglycemia.

[0018] To verify the efficacy of the promoter of the invention, in a model system more similar to human diabetes mellitus, a virus containing an insulin transgene driven by our promoter was administered to BB Wor rats. Following the onset of autoimmune diabetes mellitus, BB Wor animals treated with exogenous insulin demonstrated wide fluctuations in blood glucose, and sporadic weight gain. Withdrawal of exogenous insulin injections uniformly precipitated the recurrence of ketosis, as indicated by the detection of urine ketones. In contrast, diabetic animals treated by peripheral administration of our virus maintained near normal blood glucose values for more than two months, without exogenous insulin injections. Transgene treated animals were able to tolerate a 24-hour fast without worsening hypoglycemia, and were able to normalize blood sugars following a 2gm/kg intraperitoneal glucose tolerance test within three hours.

[0019] The utility of the promoter of the invention to appropriately regulate the production and secretion of transgenic insulin from hepatocytes both *in* vitro, and *in* vivo, was demonstrated by utilizing transcription to control transgene expression. We believe that our novel, chimeric promoter possess the capacity to regulate transgenic production from liver cells in response to metabolic signals of insulin need.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] The above and other objects, novel features and advantages of the present invention will become apparent from the following detailed description of the invention, as illustrated in the drawings, in which:

[0021] Figures 1A and 1B are schematic drawings showing the origin of sequences, and the binding sites for known DNA-binding proteins, comprising the chimeric $(GIRE)_nBP$-1 promoters, and construction of $(GIRE)_nBP$-1 promoter driven luciferase plasmids;

[0022] Figure 2 is a graphical illustration of glucose responsiveness of $p(GIRE)_2BP$-1Luc as determined by transient transfection assay;

[0023] Figure 3 is a graphical illustration showing comparative response of $p(GIRE)_nBP$-1Luc constructs to stimulation by glucose;

[0024] Figure 4 illustrates comparison of insulin dose response curves for $p(GIRE)_2BP$-1Luc performed with lactate or glucose;

[0025] Figure 5 is a graphical illustration showing time course of $(GIRE)_4$BP-1 promoter driven protein production;

[0026] Figure 6 is a graphical illustration of a glucocorticoid response wherein hepatocytes transfected with p$(GIRE)_2$BP-1Luc were exposed to a serum-free medium containing insulin 10^{-9}M, with dexamethasone and glucose as indicated;

[0027] Figure 7 is a graphical illustration of glucagon effects on $(GIRE)_n$BP-1 promoter/enhancers;

[0028] Figure 8 illustrates post-translational insulin processing in primary cultured hepatocytes;

[0029] Figure 9 is a graphical illustration of immunoreactive insulin secretion from hepatocytes;

[0030] Figure 10 is a graphical illustration of determination of biological activity for hepatocyte produced insulin;

[0031] Figure 11 illustrates glucose stimulation of $(GIRE)_3$BP-1 2xfur mediated human insulin expression in hepatocytes;

[0032] Figure 12 is a graphical illustration of glucose responsive stimulation of insulin secretion from hepatocytes in primary culture;

[0033] Figures 13A and 13B illustrate the effect of glucose, and its withdrawal, on insulin secretion from stably transfected H4IIE hepatoma cells;

[0034] Figure 14 is a graphical illustration of glucose responsive secretion of human insulin by primary cultured rat hepatocytes;

[0035] Figures 15A and 15B illustrate glucose stimulated human insulin expression following Ad/(GIRE)$_3$BP-1 2xfur administration *in vivo*;

[0036] Figures 16A and 16B illustrate blood glucose and body weight response to graded dose administration of Ad/(GIRE)$_3$BP-1 2xfur;

[0037] Figures 17A and 17B illustrate daily weights of sham treated animals, or diabetic animals treated with Ad/(GIRE)$_3$BP-1 2xfur;

[0038] Figure 18 illustrates mean daily blood glucose values for sham-treated, and Ad/(GIRE)$_3$BP-1 2xfur-treated rats made diabetic with low-dose (120-125mg/kg) STZ ;

[0039] Figure 19 is a histogram of random blood glucose values for Ad/$(GIRE)_3$BP-1 2xfur-treated rats;

[0040] Figures 20A and 20B illustrate blood glucose values for sham-treated, and Ad/$(GIRE)_3$BP-1 2xfur-treated rats made diabetic with high-dose (290mg/kg) STZ ;

[0041] Figures 21A and 21B illustrate the effect of IPGTT on blood glucose in normal and diabetic rats treated with Ad/$(GIRE)_3$BP-1 2xfur;

[0042] Figure 22 illustrates fasting tolerance of Ad/$(GIRE)_3$BP-1 2xfur-treated STZ-diabetic rats;

[0043] Figure 23 illustrates that Ad/$(GIRE)_3$BP-1 2xfur treatment ameliorates hyperglycemia in spontaneously diabetic BB Wor rats;

[0044] Figure 24 illustrates that Ad/$(GIRE)_3$BP-1 2xfur treatment induces weight gain in a spontaneously diabetic BB Wor rat;

[0045] Figure 25 illustrates that weight gain in Ad/$(GIRE)_3$BP-1 2xfur treated diabetic BB Wor rats is intermediate between the abnormally low

weight gain of SQ Insulin treated rats, and the excessive weight gain of diabetic rats treated with continuous release Linshin insulin pellets;

[0046] Figures 26A and 26B illustrate that Ad/(GIRE)$_3$BP-1 2xfur treated diabetic BB Wor rats withstand prolonged chow deprivation without progressive hypoglycemia, and lose less weight than normal, non-diabetic animals; and

[0047] Figure 27 illustrates that blood sugar of Ad/(GIRE)$_3$BP-1 2xfur treated diabetic BB Wor rats returns to baseline within 140 minutes following an intraperitoneal glucose challenge (2gm/kg).

DETAILED DESCRIPTION OF THE INVENTION

[0048] We hypothesized that transcriptional regulation of transgenic insulin production would be feasible, but that to be successful, insulin expression should be restricted to a single tissue, must be sufficient to fulfill metabolic requirements, and must be sufficiently regulated to both avoid lethal hypoglycemia, and accommodate glucose loads. To satisfy these requirements, a family of chimeric promoters composed of the Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) basal promoter, and multimers of the glucose response element (GIRE) of the liver-pyruvate kinase (L-PK)

promoter, was designed and constructed. IGFBP-1 was chosen for an expression largely restricted to the liver, its robust production, and characteristics of its metabolic regulation (References 19-25).

[0049] The liver possesses a prodigious synthetic capacity, is readily accessible as a site for viral gene transfer, and is the first recipient of nutrient blood flow from the intestinal tract. This location provides a theoretical advantage in the early detection of glucose influx into the circulatory system. Moreover, the liver is also the only extra-pancreatic tissue that expresses proteins critical to the metabolic sensory mechanism known to control insulin secretion from β-cells, the GLUT-2 protein, and the high K_m hexokinase, glucokinase (References 26-27). In the liver, the IGFBP-1 promoter mediates a robust production of IGFBP-1. Densitometric scanning of Western ligand blots indicates a stimulated secretion of rIGFBP-1 from cultured hepatocytes of ~120 μg/24 hr/3.6x10^6 cells, or 10 x 10^{-16} mol/ 24 hr/cell (Reference 28). Such calculations suggest that only a small percentage of hepatocytes would be required to produce insulin to provide circulating physiologic levels. Yet the expression of IGFBP-1 is tightly regulated at the level of transcription (Reference 25). Glucocorticoids stimulate IGFBP-1 gene expression, as do stimulators of cAMP production (Reference 23). However, these stimulated activities appear to all be subordinate to the inhibitory effects of insulin (Reference 29). Experiments using hepatocytes *in vitro* indicate that 90% of IGFBP-1 transcription is inhibited within 15 minutes of exposure to insulin, and

that this effect predominates over the stimulation produced by either cAMP, or glucocorticoids (References 30-31).

[0050] To confer stimulation by glucose to the IGFBP-1 promoter, a characteristic we believed critical to the function of an insulin transgene, a family of promoters was constructed, which are active in hepatocytes, stimulated by glucose, and inhibited by insulin. The glucose response element (GIRE) of the rat L-PK gene was inserted directly upstream of the insulin sensitive element of the rat IGFBP-1 (BP-1) basal promoter. (Figure 1A). These promoter constructs, inserted into a luciferase expression vector, vary with respect to the number of GIRE sequences present, and their orientation. For example, Figure 1B illustrates constructs with one to four repeat units of the compound GIRE. In $p(GIRE)_1BP$-1Luc and $p(GIRE)_4BP$-1Luc the L-PK sequences remain in their native orientation, whereas they are reversed in $p(GIRE)_2BP$-1Luc and $p(GIRE)_3BP$-1Luc (Figure 1B). Within each construct all GIRE sequences, share the same orientation. Like IGFBP-1, L-PK expression is restricted to the liver, and is regulated largely at the level of transcription (Reference 32). In contrast to IGFBP-1, investigators have localized a GIRE within the first 200 base-pairs upstream of the transcription start site (Reference 33). The GIRE is a compound element, comprised of an hepatic nuclear factor-4 (HNF-4) binding site, and an actual GIRE that binds the Glucose Response Element Binding Protein (Reference 34). While active as an isolated element, function of the GIRE is enhanced by juxtaposition to the

HNF-4 binding site (Reference 35). Transcriptional activation via the compound GIRE is stimulated by exposure to glucose, but not lactate or fructose, and this stimulation can be inhibited by either co-stimulation of cells with glucagon, or intracellular production of an inhibitory transcription factor, COUP-TF (Reference 35).

[0051] To validate the function of the promoters of the invention, their response to various metabolic signals of insulin need was tested. Initial observations and characterization confirming our hypothesis were obtained *in vitro* utilizing both immortalized cell lines and primary cultured hepatocytes.

Response of $p(GIRE)_n$BP-1Luc Constructs to Glucose and Insulin

[0052] As a first evaluation of the transcriptional activity of our constructs, the response of a representative promoter to both insulin and glucose *in vitro* was tested (Figure 2). Rat hepatocytes in primary culture transfected with $p(GIRE)_2$BP-1Luc, and pCMVβgal as a control for transfection efficiency, were incubated in either 10^{-10}M or 10^{-7}M insulin; concentrations known to span the range of insulin inhibitory effects with respect to the IGFBP-1 promoter *in vitro*. Reporter activity is reported as a ratio of luciferase to β-galactosidase activity. Results are the means ± SEM of three independent experiments, each with triplicate plates. When cells were provided lactate (10 mM), which does not stimulate the GIRE (Reference 35), promoter activity was

suppressed approximately 60% in the presence of 10^{-7}M insulin, indicating function of the insulin response region (IRR) within the IGFBP-1 promoter. Replacing lactate with increasing amounts of glucose resulted in a 3-fold increase in promoter activity, indicating function of the L-PK-derived glucose response elements. Each of the $(GIRE)_n$BP-1Luc constructs was inhibited by insulin, and stimulated by glucose. Glucose concentrations were chosen to span the normal range in both humans (75-140mg/dl) and rats (110-387mg/dl). The fact that glucose increased expression in the face of an inhibitory concentration of insulin (10^{-7}M) demonstrates that GIRE-mediated stimulation is not subordinate to suppression by insulin.

[0053] Glucose dose response studies were performed for each of the four promoters to determine if the number of GIRE's present affect the magnitude or kinetics of the response. Primary hepatocytes were co-transfected with the $p(GIRE)_n$BP-1Luc constructs and pCMVβgal, and parallel plates were supplied with either lactate, or increasing concentrations of glucose during an overnight incubation. Promoter response was calculated as a ratio of luciferase to β-galactosidase activity. Results are the means ± SEM from a minimum of three independent experiments, each with triplicate plates. Increasing glucose concentrations induced a progressive increase in normalized reporter activity for each of the constructs in the presence of 10^{-7}M insulin (Figure 3). While the presence of a single glucose response element was sufficient to induce a statistically significant glucose response, the

magnitude of this response increased with the copy number of GIRE's. In this series of experiments, exposing cells to 25 mM glucose stimulated expression to levels ranging from 1.6 fold for $p(GIRE)_1BP\text{-}1Luc$, to a maximum of 6.4-fold for $p(GIRE)_3BP\text{-}1Luc$, when compared to baseline. In contrast to the variability in maximal stimulation, all constructs appeared to share a similar sensitivity to glucose, and responded across the tested range of 5-25mM.

[0054] The increased expression observed following exposure to glucose is presumably due to transcriptional stimulation mediated by the GIRE. However, a glucose dependent mechanism that diminishes insulin-mediated inhibition might produce similar results. To distinguish between GIRE mediated stimulation, and inhibition of the insulin response region, hepatocytes transfected with $p(GIRE)_2BP\text{-}1Luc$ were treated with increasing amounts of insulin (10^{-11} to 10^{-6} M), and supplied with either 10mM lactate (open circles) or 25mM glucose (closed circles) as a carbohydrate source (Figure 4). Promoter response was calculated as a ratio of luciferase to β-galactosidase activity. Results are representative of three independent experiments, and show the means of triplicate samples. At all insulin concentrations reporter activity was two-three fold greater in cells exposed to glucose than in cells provided lactate. Moreover, the proportional increase in activity induced by glucose was similar across the entire range of insulin concentrations. As determined from the plotted data, the insulin concentration associated with a 50% inhibition of activity (the IC_{50}) was approximately 10^{-9}M.

Time Course of p(GIRE)$_3$BP-1Luc Response to Glucose

[0055] Time course experiments were performed to determine the kinetics of glucose-stimulated expression of a transgene protein. Hepatocytes were transfected with a p(GIRE)$_4$BP-1Luc and cultured in glucose-free medium for 24 hours in the presence of 10^{-7}M insulin. Medium was replaced with glucose-containing medium (25mM) at time 0, and cells were harvested at the times indicated. Following exposure to glucose, an increase in the quantity of transgene protein product could be detected in cells transfected with p(GIRE)$_4$BP-1 Luc, in as little as one hour, with a progressive increase over the 10 hour period examined (Figure 5). Hepatocytes were co-transfected with p(GIRE)$_4$BP-1Luc and pCMVβgal. Results are means ± SEM for triplicate plates, and are expressed as a ratio of luciferase to βgalactosidase activity.

Stimulation of a p(GIRE)$_n$BP-1Luc Construct by Glucocorticoids

[0056] Since the insulin response region within the IGFBP-1 promoter overlaps with a stimulatory glucocorticoid response element, (Reference 36), we hypothesized that glucocorticoids would also stimulate our chimeric promoter. To test this, hepatocytes in primary culture were transfected with p(GIRE)$_2$BP-1Luc, and exposed to increasing concentrations of the glucocorticoid dexamethasone, while in either low (5mM) or high

(25mM) glucose medium. Analysis of luciferase reporter gene expression demonstrated that dexamethasone stimulates the $(GIRE)_2$BP-1 promoter in a dose dependent fashion (Figure 6). Moreover, reporter activity at a given concentration of dexamethasone was greater when cells were exposed to the higher glucose concentration. Thus, glucocorticoid stimulation appears to enhance the stimulation afforded by exposure to glucose.

Glucagon Inhibits Glucose Mediated Stimulation of p$(GIRE)_n$BP-1Luc Constructs

[0057] We next evaluated the affect of glucagon on $(GIRE)_n$BP-1 mediated transcription. Glucagon increases cAMP production within hepatocytes, which may stimulate IGFBP-1 transcription via cAMP response element known to be present within its 5-prime promoter (Reference 37). However, cAMP is believed to inhibit the stimulatory affect of glucose on the L-PK promoter (Reference 38). To determine which of these conflicting effects may predominate with our chimeric promoters, hepatocytes were transfected with p$(GIRE)_2$BP-1Luc, provided medium containing either a low (5mM) or high (25mM) concentration of glucose and 10^{-9}M insulin. Parallel plates were then exposed to increasing doses of glucagon, and normalized luciferase activity measured following an overnight incubation. Advancing glucagon concentrations produced minimal increases in luciferase activity under conditions of low glucose. However, glucagon inhibited the stimulation

mediated by high glucose to a significant extent, in a dose dependent fashion (Figure 7).

Hepatocytes Produce Mature Human Insulin via a Modified Proinsulin Construct

[0058] Excision of C-peptide from translated proinsulin is a function normally restricted to tissues that express the requisite specific endopeptidases, prohormone convertase-1/3 and prohormone convertase-2 (Reference 39). To enable hepatocytes to process proinsulin to insulin, we obtained a human proinsulin cDNA (2xfur) (Reference 40), modified to allow C-peptide excision by furin, a protease expressed in many tissues including hepatocytes (Reference 41). Following construction of a CMV promoter driven plasmid incorporating the 2xfur sequence (pCMV2xfur), we transfected hepatocytes, and metabolically labeled nascent cellular proteins with [^{35}S]-methionine/cysteine. Hepatocytes were labeled for either four, or twenty four hours. Conditioned medium and cell lysates were immunoprecipitated with human insulin specific anti-serum and protein-A-sepharose, then electrophoresed on an 18% sodium-dodecyl-sulfate-polyacrylamide gel, using a 0.4M MESNA loading buffer. Molecular weight markers included the 3.6kD bovine insulin B-chain, and [^{125}I]-mono-iodinated human insulin A-chain. Electrophoresis, under reducing and denaturing conditions, of anti-insulin immunoprecipitates from both cell lysates and conditioned medium revealed a band whose intensity increased between 4 and

24 hours (Figure 8). With an apparent molecular weight of 9.8kD the band's extrapolated size is greater than either the insulin A- or B-chain in combination with the C-peptide, but similar to the 9kD determined for human proinsulin expressed from the same construct in myoblasts (Reference 42). In medium conditioned for 24 hours, immunoprecipitated bands with mobilities identical to the B- and A-chains of insulin are present (Figure 8); confirming that the insulin transgene product is processed into mature insulin, and that mature insulin is secreted into the culture medium. Independent experiments performed using non-transfected cells failed to reveal the three bands corresponding to insulin products.

[0059] Medium conditioned by hepatocytes transfected with pCMV2xfur and then assayed for human insulin provides an estimation of the capacity of hepatocytes to produce human insulin. After transfection with 1, 5, or 10μg pCMV2xfur /60mm plate, hepatocytes were washed twice in PBS and cultured overnight in serum and insulin free medium. Enzyme-immunoassay performed on an aliquot of the 3ml of medium per plate conditioned by non-transfected cells demonstrated undetectable levels of immunoactivity. In contrast, 3 ml of medium conditioned by transfected cells produced levels of insulin immunoactivity greater than 10-fold the concentration in fasting human serum (5-20μU/ml) (Reference 43) (Figure 9). The immunoassay employed reported an upper limit of detection of 300μU/ml. The detection of insulin

immunoactivity in an assay possessing minimal cross-reactivity with pro-insulin (0.005%, Abbot Diagnostics) provides further evidence of successful proinsulin processing by hepatocytes.

[0060] The biological activity of the transgene of the invention was confirmed by testing the capacity of secreted insulin to inhibit insulin-sensitive reporter gene expression in a transient transfection assay. Primary cultured hepatocytes were co-transfected with pCMVβgal, and p-324-+96BP-1Luc, an insulin suppressible luciferase expression vector driven by the rIGFBP-1 promoter. Cells were cultured overnight in serum free medium containing 10^{-7}M insulin. Control cells were provided serum free medium, which had been conditioned overnight by non-transfected hepatocytes, and to which insulin had been added at a concentration of either 0, 10^{-7} or 10^{-9}M. Test cells were supplied medium conditioned overnight by hepatocytes transfected with pCMV2xfur (5μg/60mm plate), and all cells were harvested for luciferase and β-galactosidase assay following a further overnight incubation. As expected, compared to 10^{-9}M insulin, exposure to 10^{-7}M insulin inhibits reporter expression driven by the -324-+96BP-1 promoter in control cells (Figure 10). More significantly, pCMV2xfur conditioned medium also inhibits expression, and the degree of inhibition is consistent with an insulin effect in the conditioned medium on the approximate order of magnitude of 10^{-7}M insulin.

Glucose-Stimulated Human Insulin Production in Rat Hepatocytes

[0061] We then determined if a glucose and insulin sensitive promoter could mediate glucose responsive insulin production from hepatocytes. We first created a glucose-responsive insulin transgene, $(GIRE)_3$BP-1 2xfur, by replacing the luciferase sequence in p$(GIRE)_3$BP-1Luc with the 2xfur proinsulin sequence. To verify that glucose stimulation occurred at the level of transcription, we performed Northern analysis of primary hepatocytes infected with an adenovirus containing the $(GIRE)_3$BP-1 2xfur sequence. [Ad/$(GIRE)_3$BP-1 2xfur (1×10^8 PFU, MOI ~10)]. Total RNA was isolated from transduced cells cultured overnight in serum-, and insulin-free medium containing either lactate (10mM) or varying concentrations of glucose (5 – 25mM). Membranes were sequentially probed for human insulin and rat GAPDH, and the degree of glucose stimulated insulin expression determined by dividing lane specific densitometry measurements for insulin by readings obtained for GAPDH (Figure 11). Adding glucose to the medium increased insulin expression relative to cells exposed to lactate alone ($P<0.05$), while increasing glucose from 5 to either 10 or 25mM stimulated insulin expression still further ($P<0.05$).

[0062] Hepatocyte insulin production also increased in response to glucose. Following transient transfection with p$(GIRE)_3$BP-1 2xfur (5μg/60mm plate) primary hepatocytes cultured in insulin-free medium were again provided lactate, or varying amounts of glucose (Figure 12). Insulin immunoactivity was detected in the conditioned medium of non-transfected

cells; possibly carried over from incubation with insulin containing medium. Results are the means ± SEM combined from three independent experiments. However, medium conditioned by cells transfected with the insulin transgene demonstrated a 4-fold increase in the amount of immunoreactive insulin, even in the absence of glucose. Substituting glucose for lactate in the culture medium stimulated a further dose dependent increase in insulin secretion. The human insulin specific RIA used in these analyses is reported to have minimal cross-reactivity with pro-insulin (<0.2%), supporting the conclusion that p(GIRE)$_3$BP-1 2xfur is able to mediate glucose responsive human insulin secretion from rat hepatocytes in primary culture .

[0063] Diminishing transgene expression following transient transfection, and the limited longevity of hepatocytes in primary culture, complicate analysis of extended time-course studies. To overcome these obstacles, and confirm the reversibility of glucose stimulated insulin secretion, we created an insulin secreting hepatoma cell line by stably transfecting H4IIE cells with p(GIRE)$_3$BP-1 2xfur. An insulin-secreting clone, A3, was chosen for further study, and cultured in two groups of triplicate plates in serum- and insulin-free medium. The first group initially received lactate-containing medium, while the second group initially received glucose containing medium. Each group then alternately received two periods of exposure to glucose, and two exposures to lactate. Triplicate wells of A3-cells were provided either 10mM lactate or 10mM glucose in an insulin-free medium. Medium was

exchanged daily, while the carbohydrate source was altered every two days; cells provided glucose, were changed to lactate, and *vice versa*. Medium was collected for analysis after two days in each condition, and assayed using a human insulin specific RIA. P<0.02 for (+) Glucose vs. (-) Glucose within each group. Mean $\pm$ SEM. Assay by human insulin human insulin specific RIA of conditioned medium collected after two days in each culture condition indicated that A3 cells continuously secreted small amounts of insulin under glucose-free conditions (5-10μU/ml) (Figures 13A-B). However, insulin levels in the culture medium were significantly greater during each period of glucose exposure (21-38μU/ml). Moreover, insulin secretion returned toward baseline upon glucose withdrawal, confirming the reversibility of glucose stimulation in this model. Increased insulin secretion following re-challenge with glucose indicates that reduced insulin secretion following glucose withdrawal was not diminished due to reduced cell viability. The order of exposure is also irrelevant, as insulin secretion increased in response to glucose irrespective of whether cells were exposed first to glucose, or first to lactate.

Summary of *in vitro* Experiments

[0064] Our results demonstrate that a metabolically sensitive promoter driving expression of a modified proinsulin cDNA can regulate human insulin secretion from hepatocytes in response to glucose exposure.

By inserting multimers of the rat L-PK glucose responsive element into the insulin-sensitive IGFBP-1 basal promoter we created a family of promoters whose activity is stimulated by glucose and inhibited by insulin. Because our chimeric promoter/enhancer constructs are novel, we chose to verify the efficacy of individual promoter elements. The results of transient transfection experiments confirm that the GIRE, the IGFBP-1 basal promoter, and its IRR, each remain functional. Exposing transfected cells to glucose produces a dose dependent increase in reporter gene expression at all insulin concentrations (Figure 2, 3 and 4). While glucose treatment of hepatocytes could be argued to increase gene expression in a non-specific manner, glucose stimulation of our promoters remains significant when expression is controlled for by co-transfected β-galactosidase activity. With respect to the IRR, insulin inhibits expression of a representative promoter in both the presence and absence of glucose (Figure 4). These findings appear to exclude the possibility that glucose exposure merely interferes with the suppressive effects of insulin, and are consistent with the fact that such an effect is also not observed with the native IGFBP-1 promoter. Furthermore, a glucose-mediated impairment of insulin inhibition would be expected to produce a shift in insulin sensitivity, and this is not observed. The IC_{50} for insulin suppression is the same in cells exposed to either lactate or glucose, and is similar to that reported for the native rIGFBP-1 promoter (Figure 4) (Reference 44). Thus, the IRR and the GIRE function independently of one

another. The fact that insulin-inhibition in the $(GIRE)_nBP$-1 system does not dominate the stimulatory effects of glucose on transgene expression is noteworthy, since stimulators of the native IGFBP-1 promoter are subordinate to the inhibitory effects of insulin (References 45-46). The concentration of glucose at which stimulation becomes measurable, resides within a range typical of normal rats, from 5 to 9 mM, and appears continuous through values commonly found in rats made diabetic by STZ administration (References 30 and 47). Moreover, stimulation increases continuously throughout the tested range of glucose concentrations (Figure 3).

Ad/$(GIRE)_3BP$-1 2xfur Transduced Hepatocytes Secrete Human Insulin *In Vitro*

[0065] To confirm the applicability of the data obtained from *in vitro* experiments in animal models of diabetes mellitus we first developed an adenoviral vector containing an insulin transgene driven by our invention. The capacity of the Ad/$(GIRE)_3BP$-1 2xfur adenoviral vector to confer metabolic responsive insulin production was verified *in vitro* by infecting rat hepatocytes in primary culture. Following overnight incubation in a glucose-free medium containing lactate (10mM), hepatocytes transduced with Ad/$(GIRE)_3BP$-1 2xfur (MOI=10) were provided a medium again containing lactate (10mM), or increasing concentrations of glucose, as shown. Conditioned medium was tested for the presence of human insulin by RIA after an additional overnight

incubation. Results are means ± SEM of triplicate plates, and are representative of two independent experiments. Insulin in the conditioned mediumincreased in response to increasing glucose exposure. Confluent hepatocytes in a 60mm dish provided lactate alone secreted 9.29 ± .71 SEM mU/ml immunoreactive insulin. In contrast, cells exposed to 30mM glucose produced an average of 46.76 ± 4.26 SEM mU/ml insulin during the same overnight incubation (Figure 14).

[0066] The correlation between glucose and insulin secretion was dose dependent, with an ED_{50} of approximately 20mM glucose, and achieved a maximum between 30 and 40mM glucose.

Ad/(GIRE)$_3$BP-1 2xfur Administration Produces Glucose Responsive Hepatic Insulin Secretion *in vivo*

[0067] With a functional adenoviral vector in hand, we verified its ability to confer glucose responsive hepatic insulin expression in animals made diabetic with (streptozotocin) STZ, a β-cell toxin. Two to four days following intravenous injection of STZ (120-125mg/kg) rats received a portal system injection of either Ad/(GIRE)$_3$BP-1 2xfur (2-3.9x10^9 PFU), or an equivalent quantity of adenoviral vector without a transgene (Ad*dl312*). Exogenous insulin treatment was continued for 2-6 days, and then discontinued. Animals were sacrificed while receiving no exogenous insulin.

[0068] Following portal system administration of Ad/(GIRE)$_3$BP-1 2xfur to STZ-treated rats , hepatic transgene expression was verified by RT-PCR. In reactions using primers specific for GAPDH, amplification of total liver RNA revealed a 300bp fragment in three animals treated with Ad/(GIRE)$_3$BP-1 2xfur, and two animals treated with the empty adenoviral vector Ad *dl312* (Figure 15A). However, insulin specific primers produced a 356bp fragment only in reactions containing RNA from Ad/(GIRE)$_3$BP-1 2xfur-treated livers. Reactions using RNA from Ad *dl312* injected livers failed to produce this band.

[0069] We confirmed transgenic protein production, and glucose responsiveness of the human insulin transgene, by measuring immunoreactive human insulin levels in three Ad/(GIRE)$_3$BP-1 2xfur-treated rats both before, and after glucose administration (Figure 15B). Following overnight access to food *ad libitum*, chow was withheld for five hours before animals received intraperitoneal injections of 3cc 50% glucose. Blood sugar was determined immediately before, and at one and two hours, after glucose administration. Following a five-hour fast the average blood glucose in these diabetic rats was 100mg/dl. As expected, glucose administration increased serum blood sugar levels, to an average 213mg/dl at one hour. However, by two hours this value had again fallen to an average of 83mg/dl. Thus, two hours after an intraperitoneal injection of glucose the average blood sugar was below that produced by a five hour fast (P=0.04). Serum levels of immunoreactive human insulin averaged 199μU/ml at time 0, and increased in response to

glucose stimulation to 449μU/ml at two hours (P=0.007 compared to pre-glucose). The serum of normal, untreated rats tested with this assay produced readings of 6.13 $\pm$ 1.00 μU/ml (n=3, mean $\pm$ SEM).

Dose Ranging Study for Ad/$(GIRE)_3$BP-1 2xfur Administration

[0070] To determine which transgene quantities might be effective in controlling glycemia and weight loss due to diabetes, we administered increasing doses of Ad/$(GIRE)_3$BP-1 2xfur to five animals made diabetic with STZ (125 mg/kg). Viral dose ranged from 0 (NaCl 0.9%) to $3.6x10^9$ PFU per animal, and all animals were sustained with exogenous insulin injections for one to six days. Glycemic control in all animals was erratic during the period of exogenous insulin administration. Following discontinuation of injected insulin, random blood sugars tended to vary inversely relative to the administered viral dose. Blood glucose in the control animal increased to >250mg/dl, and the animal developed ketonuria, weight loss, and died within two days (Figure 16A). Blood sugars in animals receiving either $3.6x10^8$ or $8.9x10^8$ PFU also remained consistently greater than 250mg/dl. In contrast, random glucose values in the animal receiving $1.8x10^9$ PFU fell to less than 200mg/dl for five days following discontinuation of insulin injections, and were less than 200mg/dl in 25 of 29 (86%) consecutive random measurements in the animal receiving $3.6x10^9$ PFU (Figure 16A). The animal

receiving this highest viral dose, maintained glycemic control for a total of 44 days before developing persistent hyperglycemia.

[0071] Changes in percent body weight also varied, but in direct relation to viral dose. Animals receiving the two highest viral doses ($1.8x10^9$ and $3.6x10^9$ PFU) steadily gained weight following discontinuation of exogenous insulin. The animal receiving $3.6x10^8$ PFU also gained weight at a diminished rate. The animal receiving $8.9x10^8$ PFU failed to recover weight lost following STZ-injection (Figure 16B).

Ad/(GIRE)$_3$BP-1 2xfur Treatment Ameliorates Metabolic Abnormalities of STZ-Induced Diabetes Mellitus

[0072] To determine if transgenic insulin production is sufficient to sustain diabetic animals following withdrawal of exogenous insulin we analyzed data from fourteen animals made diabetic by the injection of 120-125mg/kg STZ, including the animal receiving $3.9x10^9$ PFU in the dose ranging study. Changes in body weight, and blood glucose of animals treated with Ad/(GIRE)$_3$BP-1 2xfur ($3.4x10^9$-$1.4x10^{10}$PFU), were compared to animals injected with saline, or the Ad*dl312* vector alone. All animals were supported for two to six days with exogenous insulin injections.

[0073] All animals lost weight following STZ injection. Weight loss slowed, or reversed, with the initiation of exogenous insulin injections.

Among sham-treated animals weight gain was temporary. Discontinuation of insulin was followed by four days of weight gain, with subsequent stabilization in three Ad*dl312*-treated animals (Figure 17A). A fourth Ad *dl312*-treated animal, and a NaCl-treated animal failed to gain weight, while the remaining NaCl-treated animal precipitously lost weight (Figures 17A). Intake and output of the four Ad *dl312*-treated rats was measured in metabolic cages during six hours of a light period. Average chow consumption was increased 9-fold compared to normal animals. Water intake was 17-fold greater in Ad *dl312*-treated rats, than in normal animals, while output of stool and urine were increased 2.5 and 9-fold, respectively. In contrast, all Ad/$(GIRE)_3$BP-1 2xfur-treated animals continuously gained weight during a comparable time span, without the plateau observed in sham-treated animals (Figure 17B). Moreover, weight gain in Ad/$(GIRE)_3$BP-1 2xfur-treated animals continued until sacrifice (14-80 days).

[0074] In both treatment groups, average daily blood sugars rose sharply following STZ administration, and remained elevated in spite of exogenous insulin administration (Figure 18). After STZ administration rats received a portal system injection of Ad/$(GIRE)_3$BP-1 2xfur (n=8), Ad*dl312* (n=3), or NaCl 0.9% (n=2). All animals received chow and water *ad libitum*, and exogenous insulin was administered from 2-6 days. Blood glucose values were averaged across groups. For days in which multiple glucose values were available, the first value of the day was utilized. One control animal died,

and three subject animals were sacrificed during the study. Blood sugars obtained after animals again developed hyperglycemia (>250mg/dl for $\geq$3 consecutive values) were excluded from analysis. Each data point represents the contribution of at least three animals. Results are means $\pm$ SEM.

[0075] Excluding the first 24 hours following discontinuation of exogenous insulin, all blood sugar values of 6 sham-treated animals, except one, were greater than 200mg/dl. In contrast, levels of blood sugar in Ad/(GIRE)$_3$BP-1 2xfur-treated rats fell within two to four days after viral injection.

[0076] The duration of metabolic control produced by treatment with Ad/(GIRE)$_3$BP-1 2xfur was variable, lasting from 7.9 days post-viral injection to 84.9 days post-viral injection. Thereafter, all Ad/(GIRE)$_3$BP-1 2xfur-treated animals redeveloped persistent hyperglycemia (>250mg/ml for $\geq$3 consecutive measurements). As each animal developed persistent hyperglycemia, the duration of metabolic control was recorded, and elevated blood glucose values were excluded from the calculation of group averages. Sacrifice of three animals during the study reduced the number of animals contributing to daily averages. However, each data point in Figure 18 utilizes the results of at least three independent animals.

[0077] Mean blood glucose values were significantly lower in Ad/(GIRE)$_3$BP-1 2xfur-treated animals than in sham-treated animals (P<0.05 for 9 of 10 days immediately following discontinuation of exogenous insulin),

but fluctuated widely. To obtain a more detailed evaluation of efficacy we examined the frequency distribution of random blood glucose values from Ad/(GIRE)$_3$BP-1 2xfur-treated animals made diabetic with 125mg/kg STZ. Values obtained within 18 hours of exogenous insulin, or after development of sustained hyperglycemia were excluded (Figure 19). Random blood glucose values obtained for Ad/(GIRE)$_3$BP-1 2xfur-treated rats were partitioned into five ranges. Of the 186 values available for analysis, 128 (68.81%) fell between 70-200mg/dl, while 20 values (10.75%) were $\geq$ 200mg/dl, and 38 values (20.43%) were $\leq$ 70mg/dl. Only 6 values (3.23%) were $\geq$ 250mg/dl, and a single value exceeded 300mg/dl. Severe hypoglycemia, arbitrarily defined as $\leq$50mg/dl, was detected 8 times (4.30%), with a nadir of 29mg/dl. No animal died of hypoglycemia.

Ad/(GIRE)$_3$BP-1 2xfur Treatment Ameliorates Hyperglycemia Induced by High-Dose STZ

[0078] To reduce the possibility that residual endogenous insulin production had contributed to glycemic control we sought to increase β-cell destruction by increasing the dose of STZ used to induce diabetes from 125 to 290mg/kg. Seven STZ-treated rats received a portal system injection of Ad/(GIRE)$_3$BP-1 2xfur (n=5), or Ad*dl312* (n=2). All animals received chow and water *ad libitum*, and exogenous insulin was administered for two to six days.

Blood glucose increased in both animals destined for treatment with Ad/(GIRE)$_3$BP-1 2xfur, and sham treatment following STZ administration, and fluctuated widely during treatment with subcutaneous insulin. Individual blood glucose values are depicted for sham-treated rats. Upon discontinuation of exogenous insulin, these two animals developed ketonuria, precipitously lost weight, and were euthanized (Figure 20A). In contrast, the blood sugars in each of the five Ad/(GIRE)$_3$BP-1 2xfur-treated animals stabilized at levels generally less than 200mg/dl (Figure 20B). Blood glucose values for Ad/(GIRE)$_3$BP-1 2xfur-treated animals are shown as a daily average. For days in which multiple glucose values were available, the first value of the day was utilized. Blood sugars of animals that again developed hyperglycemia (>250mg/dl for $\geq$3 consecutive values) were excluded from analysis. Each data point represents the contribution of at least three animals. Results are means $\pm$ SEM.

[0079] We obtained 207 random glucose values later than 18 hours after the last subcutaneous insulin injection, but before the redevelopment of sustained hyperglycemia. (Figure 19) Of these, 103 values (49.76%) fell within the 70-200mg/dl range, 40 (19.32%) were $\leq$70mg/dl, and 14 (6.76%) were $\leq$50mg/dl. An additional 37 values (17.87%) were $\geq$200mg/dl, with 13 (6.28%) $\geq$250mg/dl. As in the previous group, no animal died of hypoglycemia.

[0080] Intraperitoneal glucose-tolerance tests, and ten-hour fasts were used to determine the response of the insulin transgene to environmental variables. After overnight feeding *ad libitum* chow was withheld for 4.5 hours from each of three rats first made diabetic by high dose (290mg/kg) STZ, and then treated with Ad/$(GIRE)_3$BP-1 2xfur. Three normal animals were used as controls. An intraperitoneal glucose tolerance test (IPGTT) was performed by administering 1.35 gm/kg glucose. Prior to injection blood glucose values in normal animals ranged from 71-94 mg/dl (Figure 21A). They increased significantly by one hour ($P<0.05$, for averages), and returned to baseline by two hours. Baseline blood glucose values in the STZ-treated animals ranged from 71-101mg/dl (Figure 21B). Thirty-minute blood glucose values were uniformly elevated, and achieved a maximum in two animals. In these two animals one-hour values had declined, and were less than 140mg/dl by two hours. All values were between 80-140mg/dl by three hours, and continued to decline by hour-4.

[0081] While no transgene-treated animal succumbed to hypoglycemia, sporadic low blood glucose values suggested the potential for over-production of transgenic insulin. To test the ability of treated animals to withstand food deprivation, the three treated animals used in the IPGTT study, were subjected to a more prolonged period of food deprivation. Following overnight access to chow *ad libitum*, serial blood glucose measurements were obtained while animals were subjected to a ten-hour fast (Figure 22). Blood

glucose values at the beginning of the fast averaged 91.3 mg/dl. Two of the a.m. glucose values were below normal for fed rats (44 and 51 mg/dl) (Reference 48). However, upon withdrawal of chow, blood glucose for these two animals increased over the next five hours, and stabilized within a normal range. The highest blood glucose fell sharply within the first 30 minutes, and subsequently stabilized at approximately 70 mg/dl for the last three hours of the fast.

[0082] We first verified that *Ad*/(GIRE)$_3$BP-1 2xfur treatment produces a significant, sustained improvement in blood sugars in rats made diabetic with streptozotocin (STZ), a β-cell specific toxin. Following induction of diabetes by administration of 125mg/kg STZ, diabetic animals were dosed with an empty adenoviral vector, or *Ad*/(GIRE)$_3$BP-1 2xfur, an adenovirus containing an insulin transgene driven by our invention. 69% of all random blood glucose measurements obtained before the recurrence of sustained hyperglycemia were between 70-200mg/dl. Moreover, rates of hypoglycemia were less than observed in two published models of aggressive treatment with exogenous insulin. In a study comparing subcutaneous insulin algorithms for the treatment of STZ-diabetic rats the lowest reported incidence of hypoglycemia was 31% $\leq$ 70mg/dl, and 16% $\leq$ 50mg/dl, but was attained only with twice daily insulin administration (Reference 49). To achieve normal 24-hour serum insulin profiles in diabetic Wistar rats, Koopmans, et al used continuous intravenous infusion and programmed meals (Reference 50).

However, even with this elaborate design, they observed severe hypoglycemia ($\leq$ 58/mg/dl) in 40% of fasting blood glucose values (Reference 50). By contrast, we observed hypoglycemia of $\leq$ 70mg/dl, or $\leq$ 50mg/dl, in 20% and 4% of all random blood sugars, respectively, without exogenous insulin administration. The degree of metabolic stability produced by our system of regulated transgenic insulin production is further underscored by the capacity of treated animals to tolerate the divergent stresses of glucose loading, and fasting. Following an intraperitoneal glucose load, blood sugars of treated-diabetic rats had fallen to baseline within 3-4 hours, and serum glucose remained stable during a 10 hour period of fasting.

[0083] Pancreatic β-cells are known to under go limited regeneration following STZ treatment (Reference 51). However, measured immunoreactive rat C-peptide in serum of STZ-treated rats (125mg/kg) was in the range for normal fasting animals, i.e. was inappropriately low for hyperglycemic animals. Reducing the contribution of endogenous insulin production by increasing the dose of STZ from 125 to 290mg/kg produced minimal changes in glycemic control, and even with this higher STZ dose 49.76% of random glucose values were between 70-200mg/dl. The percentage of random blood glucose values $\leq$50mg/dl increased from 4% in the 125mg/kg STZ group to 7% suggesting a diminished effect of endogenous insulin, but remained less than some aggressive exogenous insulin regimens (References 49-50).

Treatment with Ad/(GIRE)$_3$BP-1 2xfur ameliorates hyperglycemia in diabetic BB Wor rats

[0084] To verify the efficacy of our transgene in a model more representative of human disease, we administered the same adenoviral constructs utilized in the STZ-rat model to BB Wor rats. Diabetes prone (DP) BB Wor rats develop an autoimmune insulitis similar to that seen in human type 1 diabetes mellitus (Reference 52). Between 60 to 80 days of age animals spontaneously become hyperglycemic, fail to gain weight, and left untreated, develop lethal ketoacidosis. Each of twelve DPBB Wor animals were allowed to become diabetic (indicated by whole blood glucose measurements of $\geq$250mg/dl on two consecutive days). Following a variable period of treatment with subcutaneous insulin injections ranging from 6 to 29 days, each rat received a jugular venous injection of Ad/(GIRE)$_3$BP-1 2xfur. Viral doses ranged from 1.3 to 4 x 10^{10} PFU/kg. Within 48 to 60 hours of viral administration exogenous insulin administration was discontinued, and blood glucose levels fell below levels typically sustainable using subcutaneous insulin (Figure 23). Random blood glucose values were obtained from tail blood using a standard home blood glucose monitor. Results are means $\pm$ SD for the first daily value. Values from at least three animals contribute to each data point. Blood glucose levels remained depressed despite the cessation of exogenous insulin injections. Moreover, weight gain, which had initially stalled

with the onset of hyperglycemia resumed its normal increase following viral administration (Figure 24). While random blood glucose values were often below normal, no animal succumbed to hypoglycemia. As typified by the a representative animal weight gain plateaus, then reverses, as the animal becomes diabetic during the 8 days prior to administration of Ad/(GIRE)$_3$BP-1 2xfur. Following adenoviral administration, weight gain resumes, despite the fact that exogenous insulin administration has been stopped. In experimental groups twice daily administration of injected insulin (SQ) induced sub-normal weight gain (n=4), while treatment with sustained-release subcutaneous insulin (Linshin) pellets induced mildly excessive weight gain (n=3). (Figure 25) Weight gain in diabetic, Ad/(GIRE)$_3$BP-1 2xfur treated DPBB Wor rats (n=12) was essentially normal, andintermediate to SQ and Linshin treated animals.

[0085] The ability of treated animals to tolerate the metabolic demands of food deprivation, and overfeeding were tested by subjecting them to both a prolonged (24-hour) fast, and an intraperitoneal (IP) glucose tolerance test. All twelve treated diabetes prone (DP)BB Wor rats, and six diabetes resistant (DR)BB Wor rats were deprived of access to food and feces at the beginning of the dark cycle. Weight and blood glucose measurements were then obtained every two, for the following 24 hours. Average blood glucose values of all twelve treated animals tended to decline during the first four hours of the fast, and then stabilized. (Figure 26A). Results are means $\pm$

SD for twelve treated DPBB Wor and six non-diabetic DRBB Wor rats. Differences in means failed to reach statistical significance at all but a single time point. Continuously declining body weights measured during the fast confirmed that animals were calorie deprived (Figure 26B). Despite equivalent average body weight at onset of fasting (P=0.775) non-diabetic DRBB Wor rats progressively lost a greater percentage of body weight than Ad/(GIRE)$_3$BP-1 2xfur treated, diabetic DPBB Wor rats. This difference was significant at all time points, and persisted throughout the observation period. Water intake, and urine output was not different between groups during the same period.

[0086] Three diabetic animals treated with the insulin transgene were also subjected to an IP glucose tolerance test. Following the administration of glucose (2gm/kg) the blood sugar of each animal increased. (Figure 27) However, similar to observations in STZ-treated rats, blood glucose values had returned to baseline levels within 140 minutes. Thereafter, they either continued to decline (one animal) or stabilized (two animals).

[0087] In summary, these data confirm, and extend the findings obtained in STZ-treated rats. The spontaneous diabetes mellitus that occurs in DP BB Wor rats is ameliorated following treatment with an insulin transgene driven by our glucose and insulin responsive promoter. Following treatment with the transgene diabetic animals maintain near normal blood sugars, gain weight, and avoid ketoacidosis, all without exogenous insulin. Weight gain in

transgene treated animals is less than in animals treated with Linshin continuous-release pellets. Moreover, treated animals are able to tolerate extremes of metabolic demands without adverse events. They were able to sustain acceptable blood glucose levels throughout a prolonged fast, and lost less weight during a fast than non-diabetic animals. They were also able to return blood glucose levels to baseline values within 140 minutes following a large glucose load.

[0088] The following materials and techniques were used in the present invention.

Promoter and Viral Constructs

[0089] Base pairs –324 - +96 of the rat IGFBP-1 promoter were PCR amplified from p-930bpcat (Reference 44), and the product ligated into a TA-cloning vector, pCRII™ (Invitrogen, Carlsbad, Ca.). The primers were as follows, novel restriction sites (*Mlu*-I in the forward primer, and *Nhe*-I in the backward primer) are underlined: forward; 5'-GCG<u>ACGCGT</u>TCCCTTAGGTATTCCTTGAGT-TCGG-3', backward; 5'-GCG<u>GCTAGC</u>TAGTAGCGGAAGTGGTGGTTCACAG-3'. A 526 bp *Kpn*-I / *Xho*-I restriction fragment was then directionally inserted into the luciferase expression vector, pGL2Basic (Promega, Madison, WI), to create p-324-+96BP-1Luc. The p(GIRE)$_n$BP-1Luc plasmids were constructed by inserting copies of the glucose responsive element from the rat L-PK gene into the IGFBP-1

promoter. Oligonucleotides corresponding to 50 bp of the positive (5'-GGGCGCACGGGGCACTCCCGTGGTTCCTGGACTCTGGCCCCCAGTGT-A-3') and negative (5'-ATGTACACTGGGGGCCAGAGTCCAGGAACCACGG-GAG-TGCCCCGTGCGCCC-3') strands of the rat L-PK GIRE sequence were annealed, multimerized, and size fractionated by polyacrylamide electrophoresis. DNA representing a sequence of GIRE multimers, from one to four, was isolated from excised bands, and blunt-ended by treatment with Klenow. Insertion of GIRE multimers into *EcoICR*-I restricted p-324-+96BP-1Luc removed all IGFBP-1 sequences 5' to bp –114, and resulted in placement of GIRE sequences immediately 5' to the IGFBP-1 insulin responsive region. Sequencing of resultant plasmid constructs unexpectedly revealed uniform multimerization of all GIRE elements in a head-to-tail orientation. Blunt-end ligation with p-324-+96BP-1Luc consequently produced constructs in which all GIRE's were either in the native, or reverse orientation.

[0090] An adenoviral vector expressing human insulin, Ad/(GIRE)$_3$BP-1 2xfur, was constructed using the Adeno-Quest kit per manufacturer's instructions (Quantum Biotechnologies, Inc., Montreal, Canada). A *Sal*-I/*Hinc*-II fragment of p(GIRE)$_3$BP-1 2xfur containing the glucose and insulin sensitive promoter coupled to the 2xfur sequence was inserted into the transfer vector pQBI-AdBN . Homologous recombination following co-transfection of pAdBN- (GIRE)$_3$BP-1 2xfur and manufacturer-supplied viral DNA into HEK-293 cells permitted isolation of E1a/E1b/E3

deleted adenovirus containing the insulin transgene. We verified the capacity to induce insulin production by screening medium conditioned by primary cultured hepatocytes infected with crude lysates of expanded viral plaques using a human-insulin specific RIA (Linco Research, St. Charles, MO). Following three-fold plaque purification, viral preparations were purified by double CsCl density-gradient centrifugation, dialyzed against 10%glycerol/HBS pH 7.4, aliquoted, and stored in this same buffer at -70^{0}C. Viral concentration was determined by the tissue-culture infectious dose method ($TICD_{50}$).

Sequencing

[0091] Sequencing of chimeric promoters was performed in the DNA Core Facility of Atlanta VA Medical Center/Emory University, using an Applied Biosystems Model 377 automated sequencer (Perkin Elmer, Foster City, CA).

Cell Culture and Transfection

[0092] Hepatocytes were isolated from male Sprague-Dawley rats (150 to 200g, Charles Rivers Laboratories, Wilmington, MA) by a modification of the collagenase (Worthington, Freehold, NJ) perfusion method of Seglen (Reference 53). Briefly, following anesthesia by intra-peritoneal

injection of a ketamine/xylazine mixture, the portal vein was canulated with a 20 gauge venous catheter, and the liver perfused with collagenase-buffer for six minutes. Livers were removed *en mass*, and the cells gently shaken from the perforated hepatic capsule in DMEM/F12 (Mediatech Cellgro, Herndon, VA) supplemented with 10% fetal calf serum (FCS) (Atlanta Biological, Marietta,GA). Hepatocytes (3.5-5 x 10^6 cells) were added to 60mm plates (Falcon/Becton Dickinson Labware, Lincoln Park, NJ) coated with rat tail collagen (Sigma, St. Louis, MO), and non-adherent cells removed twenty minutes later. Incubations were performed in an atmosphere of 95% air, 5% CO_2 at 37^0 C. Transient transfection (5μg of test construct and 0.1 or 0.2μg pCMVβ-gal per plate as a control for transfection efficiency) was performed overnight in 3%FCS/DMEM/F12 using a DNA/$CaPO_4$ co-precipitation method, modified from Ginot et al (Reference 54). Plasmid DNA was isolated by twice banding over a CsCl gradient, followed by extensive dialysis against TE buffer. Hepatocyte response to glucose was evaluated by providing serum-free DMEM/F12 medium (JRH Biosciences, Lenexa, KS) custom blended without glucose, to which glucose or L(+)-lactate were subsequently added. To control for osmotic forces, carbohydrate concentration was maintained by substituting equimolar amounts of L(+)-lactate for glucose, up to 10 mM. Reconstituted, defined medium contained amino acids at 20x concentration of rat arterial plasma, dexamethasone 10^{-7}M (Sigma), PCN/Strep [100U/ml PCN, 100mcg/ml streptomycin](Sigma), human apo-transferrin 0.01mg/ml (Sigma),

and fatty-acid free, bovine serum albumin 1mg/ml (Boehringer Mannheim Biochemicals, Indianapolis, IN). Bovine insulin (GibcoBRL, Gaithersburg, MD), was added from a sterile filtered, 10^{-4}M stock, dissolved in 0.1M HCl. D+Glucose was added from a 140mg/ml sterile filtered stock solution. L(+)-lactate (Sigma) was dissolved into defined medium just before addition to cells. Stable transfection was performed as described (Bonifacino,J. Current Protocols in Molecular Biology, 1997, J.Wiley, and Sons). Briefly, 70% confluent H4IIE cells (a generous gift of Dr. John Koontz, University of Tennessee) were co-transfected with p(GIRE)$_3$BP-12xfur (2μg/60mm plate) and pRcCMV/Neo (Invitrogen, Carlsbad, CA) (2μg/60mm plate), using the adenovirus/polyethylenimine/DNA-conjugate method (References 55). Colonies were isolated under selection in G418 (0.3 mg/ml) (GibcoBRL, Gaithersburg, MD), and screened for human insulin production by human insulin specific RIA (Linco, Inc) of conditioned medium. An insulin secreting cell line, designated A3, was utilized to document glucose dependent insulin secretion. Two groups of A3 cells expanded to 80% confluence in triplicate wells of collagen coated, 6-well plates were washed twice with PBS to remove traces of serum. Thereafter, cells were alternated between medium containing either 10mM L(+)-lactate, or 10mM glucose every two days, for eight days. Culture medium was changed daily. One group was initially provided lactate. The other group initially received glucose. Cells were washed every second

day to minimize mechanical stimulation, while reducing carry-over contamination, and conditioned medium was collected just prior to each wash.

Reporter Assays

[0093] Luciferase assays were performed using the Luciferase Reporter Assay System (Promega, Madison, WI). Twenty microliters of cell lysate, created by adding 250μl of Reporter Lysis Buffer (Promega, Madison, WI) per 60 mm plate, was combined with 100 μl of Luciferase Assay Buffer per well of a 96 well plate. Emitted light was measured for 20 seconds at ambient temperature in a Model ML 3000 plate reading luminometer (Dynatech, Chantilly, VA). β-galactosidase assays were performed using the same instrument. However, for these assays 10 μl of lysate were combined with 100 μl of Lumigal (Lumigen, Detroit, MI), and emitted light measured for 10 seconds at 37^{0}C.

Protein Labeling

[0094] Protein labeling using [^{35}S] - methionine/cysteine was performed essentially as described by Marriott, et al. (Reference 56). Freshly plated primary hepatocytes were transfected by Ca/PO_4 DNA co-precipitation, and cultured overnight in 3%FCS/DMEM/F12 (Mediatech Cellgro). Cells were washed twice in 1xPBS, and provided a methionine/cysteine depleted medium (JRH Biosciences, Lanexa, KA) for two hours before addition of 4 mCi of [^{35}S] -

methionine/cysteine (Promix, Amersham/Pharmacia Biotech, Arlington Heights,IL) per 100 mm plate. Cultures were continued for either 4 hours, or overnight, and conditioned medium collected. Cells were washed twice, and collected in 1x PBS, prior to lysis via three cycles of freeze/thaw. Conditioned media and cell lysates were immunoprecipitated with human insulin specific anti-serum (Linco Research, St. Charles, MO), and Protein-A-Sepharose CL-4B (Pharmacia Biotech). Immune complexes were subjected to denaturing SDS-PAGE using an 18% polyacrylamide separating gel, and a 4% stacking gel. MESNA (2-mercaptoethanesulfonic acid) (0.4M) (Sigma, St. Louis, MO) was included in the loading buffer to assure complete reduction of disulfide bonds. A commercial polypeptide standard, which included the bovine insulin B-chain, and [^{125}I]-labeled human insulin, mono-iodinated on the A-chain (Linco Research, St. Charles, MO), were included as positive controls for the B and A chains, respectively. After drying overnight, gels were exposed for 2-4 days, and detected using a phosphor-imager (Molecular Dynamics, Sunnyvale, CA).

[0095] Insulin in conditioned medium was measured using either a dual monoclonal antibody Microparticle Enzyme Immunoassay (MEIa) (IMx Insulin, Abbott Diagnostics, Abbott Park, IL), or a standard anti-human insulin RIA kit (Linco Research, St. Charles, MO). The manufacturer of the dual monoclonal antibody MEIa reports the dynamic range as 1-300 μU/ml, the sensitivity as greater than 1.0 μU/ml, the cross-reactivity with human proinsulin = 0.005%, within assay CV's of 4.0%, between assay coefficient of variation

4.5%, and total run coefficient of variation of 6.0%, at a mean concentration of 8.3 μU/ml. The standard RIA kit is reported to have cross-reactivities of human proinsulin <0.2%, Des 31,32 insulin <0.2%, Des 64,65 insulin = 76%, rat insulin <1.0%. Each was used according to manufacturers' instructions.

Northern Analysis

[0096] Ten micrograms of total RNA per lane, isolated from transduced primary hepatocytes using TRIzol Reagent (GibcoBRL/Life Technologies, Gaithersberg, MD), was electrophoresed on a 1%-agarose-7% formaldehyde gel. Following capillary transfer and UV cross-linking to a nylon membrane (Hybond-N, Amersham/Pharmacia Biotech, Arlington Heights, IL), blots were pre-hybridized, and hybridized at 68°C using QuikHyb (Stratagene, La Jolla, CA), and washed for 30 minutes at 68°C with a 0.1%-SSC/ 0.1% SDS-solution. Images were developed, and quantified using a phosphor-imager with accompanying densitometry software (Molecular Dynamics, Sunnyvale, CA). Following initial analysis using a human insulin probe, membranes were stripped and re-probed using a human GAPDH sequence. Probes were labeled using random priming (Prime-It II, Stratagene, La Jolla, CA) of plasmid restriction fragments derived from either pLinkIfurIIfur for insulin, or pBShGAPDH for GAPDH (a kind gift from Dr. Maria Alexander-

Bridges, Howard Hughes Medical Institute, Massachusetts General Hospital, Boston, MA).

Human Insulin, Rat C-Peptide RIA

[0097] Concentrations of human insulin in conditioned medium, or rat serum, were determined using a human insulin specific RIA reported to have less than 0.1% cross-reactivity with rat insulin, <0.02% cross-reactivity with human proinsulin, and human des 31,32 split-insulin. Cross-reactivity to human des 64,65 split-insulin is reported to be 76% (Linco Research, St. Charles, MO). Rat C-peptide was measured using an RIA with less than 1% cross reactivity to human C-peptide, human insulin, or human proinsulin (Linco Research, St. Charles, MO).

RT-PCR

[0098] Immediately upon sacrifice livers of treated and control animals were flash-frozen in liquid nitrogen, and stored at –70^{0}C. Total RNA (5μg), obtained by using Trizol (Gibco BRL, Gaithersburg, MD) per manufacturer's instructions, was subjected to reverse transcription using an oligo-dT_{17} primer, recombinant RNAsin, and Moloney- Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (all Promega, Madison,WI). M-MLV RT was inactivated at 95^{0}C, and PCR reactions were performed using a Gene

Amp PCR System 9600 thermal cycler (Perkin Elmer, Norwalk, CT), and AmpliTaq DNA Polymerase (Perkin Elmer, Norwalk, CT). The cDNA-mixture was allowed to react for 19 (GAPDH), or 22 (insulin) cycles. Primers used for human insulin were 5'-ACCATGGCCCTGTGGATGCGC-3' (forward), and 5'-CTAGTTGC-AGTAGTTCTCCAG-3" (reverse). Primers for GAPDH were 5'-CTGGTCATCAATGGGA-AAC-3' (forward), and 5'-CAAAGTTGTCATGG-ATGACC-3' (reverse).

Animals Studies

[0099] All studies were performed on male Sprague-Dawley rats, 200-300gm, housed in individual cages, and exposed to twelve-hour cycles of light and dark. All procedures were approved by the Institutional Animal Care and Use Committees of Emory University, and the Atlanta VA Medical Center. Unless otherwise specified, animals were provided water and standard rodent chow *ad libitum*.

[0100] Animals were made diabetic by intravenous administration of streptozotocin (STZ) (Pfanstiehl Laboratories, Inc., Waukegan, IL), dissolved in citrate buffer, pH 4.0. The diagnosis of diabetes mellitus was based upon findings of two or more consecutive random blood glucose values greater than 250mg/dl, and ketonuria greater than, or equal to, 3 (out of 5 possible) by urine test strip. Blood glucose was measured via the glucose

oxidase method, using tail-blood and a One-Touch Profile portable blood glucose monitor (Lifescan,Inc., Milpitas, CA). Urine pH, bilirubin, and acetoacetate were assessed using Chemstix 10 (Boehringer Mannheim, Elkhart, ID). Upon diagnosis of diabetes treatment with subcutaneous injections of insulin (U-100 Lente recombinant human insulin, Lilly Co., Indianapolis, IN, or U-40 PZI beef/pork insulin, Blue Ridge Pharmaceuticals, Greensboro, NC) was initiated.

[0101] On the second day following STZ injection, animals underwent recovery surgery for portal system injections of Ad/(GIRE)$_3$BP-1 2xfur, or sham treatment with either Ad *dl312*, or carrier alone (0.9% NaCl). Surgical anesthesia was achieved using intraperitoneal ketamine10mg/ xylazine 1mg/ 100gm body-weight. Ad/(GIRE)$_3$BP-1 2xfur-treated animals received 3.6 x 10^8 -3.6 x 10^9 PFU, Ad *dl312*-treated animals an equivalent Ad*dl312* dose determined by measurement of OD_{260} while NaCl-treated animals received injections of equivalent volume (Reference 57). The insulin injections begun upon diagnosis of diabetes mellitus were continued for from 1 to 6 days following surgery, or until signs of endogenous insulin production (accelerated weight gain, sustained elevation in urine pH and resolution of ketosis, or a tendency for hypoglycemia) became apparent. Animals were monitored daily to every third day for changes in weight, blood glucose, and urine chemistries. Serum for insulin RIA was obtained via jugular venipuncture under Metofane (Mallinckrodt Veterinary, Inc., Mundelein, IL)

induced inhalation anesthesia, and was aliquoted and stored at -20^0C prior to assay.

Statistics

[0102] Means and standard errors of the mean were calculated using programs resident in either Sigma Plot v.3.2, or Excel 97. P values were calculated using a one-tailed t-test, assuming unequal variances.

[0103] From the above, it is concluded that the invention of this application is a successful application of hepatic insulin gene therapy in two distinct rodent models of diabetes mellitus. Utilizing our chimeric glucose and insulin responsive promoter transcriptional regulation of transgenic insulin significantly improves both STZ-induced hyperglycemia, and the spontaneous hyperglycemia in DP BB Wor rats without producing lethal hypoglycemia. Additional work is contemplated to avoid unacceptably broad glucose excursion, and to develop a vector delivery system capable of allowing sustained transgene function. This may ultimately allow the extension of insulin gene therapy studies to humans.

[0104] The present invention also includes therapeutic or pharmaceutical compositions comprising a derivative of the construct of the invention in a form which can be combined with or in combination with a pharmaceutically acceptable carrier for any appropriate manner for

administration, including, for example, oral, nasal, intravenous or intramuscular administration. Appropriate dosages, duration and frequency of administration would be determined by known factors, such as the condition of the patient, the type and severity of the disease and the method of administration. The term "carrier" includes a diluent, adjuvant, excipient, or vehicle with which the peptide is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum oil such as mineral oil, vegetable oil such as peanut oil, soybean oil, and sesame oil, animal oil, or oil of synthetic origin. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

[0105] Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The therapeutic compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, capsules, powders, sustained-release formulations and the like. The composition can be formulated with traditional binders and carriers such as triglycerides. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions contain a therapeutically

effective amount of the therapeutic composition, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[0106] The composition may be formulated in accordance with routine procedures as a pharmaceutical composition adapted for local, or systemic injection or administration to human beings. Typically, compositions for local or systemic injection administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic, such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container, such as an ampoule or sachette indicating the quantity of active agent. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0107] The therapeutic or pharmaceutical compositions of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts, include those formed with free amino groups, such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups, such as those derived from sodium,

potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[0108] The present invention also provides for the modification of the derivatives of the construct of the invention such that it is more stable once administered to a subject, i.e., once administered it has a longer time period of effectiveness as compared to unmodified peptide. Such modifications are well known to those of skill in the art, e.g., polyethylene glycol derivatization (PEGylation), microencapsulation, etc.

[0109] While this invention has been described as having preferred sequences, ranges, steps, materials, or designs, it is understood that it includes further modifications, variations, uses and/or adaptations thereof following in general the principle of the invention, and including such departures from the present disclosure as those come within the known or customary practice in the art to which the invention pertains, and as may be applied to the central features hereinbeforesetforth, and fall within the scope of the invention and of the limits of the appended claims. It is further understood that the present invention is not limited to the claims appended hereto.